Detection of Kudoa septempunctata 18S rDNA in Patient Fecal Samples from Novel Foodborne Outbreaks Caused by Consumption of Raw Olive Flounder (Paralichthys olivaceus)

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Abstract

*Kudoa septempunctata* is a newly identified myxosporean parasite of olive flounder (*Paralichthys olivaceus*) and a suspected causative agent of several foodborne gastroenteritis outbreaks in Japan. Here, we report the detection of *K. septempunctata* 18S rDNA in fecal samples of outbreak patients using an efficient method based on real-time PCR. We first performed a spiking experiment to assess whether our previously developed real-time PCR assay was applicable to detect *K. septempunctata* in feces. Simultaneously, we compared the relative extraction efficacy of *K. septempunctata* DNA using three commercial kits. Finally, our detection method was validated by testing 45 clinical samples obtained from 13 foodborne outbreaks associated with the consumption of raw flounder and 41 fecal samples from diarrhea patients epidemiologically unrelated with the ingestion of raw fish. We found that the FastDNA Spin Kit for Soil (MP-Biomedical) was the most efficient method to extract *K. septempunctata* DNA from fecal samples. Using this kit, the detection limit of our real-time PCR assay was $1.6 \times 10^1$ spores per gram of feces, and positive results were obtained for 21 fecal and 2 vomitus samples obtained from the foodborne outbreaks. To our knowledge, this is the first report to describe
the detection of *K. septempunctata* DNA in patient fecal samples. We anticipate that our detection method will be useful for confirming foodborne diseases caused by *K. septempunctata* in laboratory investigations.

**Introduction**

In Japan, the number of outbreaks of an unidentified foodborne disease associated with raw fish consumption has increased since 2003 and has averaged greater than 100 per year, reaching 158 in 2010 (7). In most outbreaks, victims ingested raw olive flounder (*Paralichthys olivaceus*), but no foodborne bacteria, viruses, bacterial toxins, or chemicals were detected in the implicated foods. Symptoms of the illness occur within 2-20 h after consuming raw olive flounder and include transient, but strong, diarrhea and vomiting, with patients typically recovering within 24 h of the onset of symptoms (7). In our recent study examining the etiological agents of this foodborne disease, we conducted epidemiological and metagenomic sequence analyses, and animal experiments, which revealed that the novel myxosporean parasite *Kudoa septempunctata* was the causative agent of outbreaks (5, 7). As olive flounder is an important commercial fish and widely distributed in Japan, foodborne
outbreaks caused by *K. septempunctata* have become a notable public health concern. However, since *K. septempunctata* was first described in the trunk muscles of an aquacultured olive flounder in 2010 (10), only a limited number of investigations of this parasite have been conducted in various disciplines related to the field of food hygienics. Therefore, no effective measures for preventing *K. septempunctata* foodborne outbreaks caused by the consumption of raw flounder have developed, other than eliminating olive flounder infected with this parasite. Accordingly, to prevent the spread of *K. septempunctata* foodborne outbreaks, it is important to investigate each outbreak case in detail to develop suitable public health strategies.

Outbreaks of this novel foodborne disease are typically associated with the consumption of traditional Japanese foods, particularly ‘sushi’ (cooked rice with sliced raw-fish) and ‘sashimi’ (sliced raw fish), which are prepared using raw flounder muscle tissue containing more than 10,000 spores of *K. septempunctata* per gram. However, as certain outbreaks had low attack rates or odds ratios for raw flounder (7), the diagnosis of this foodborne disease can be difficult using only epidemiological analyses. Therefore, the detection of *K. septempunctata* in flounder consumed by patients or in patient samples is...
important for the confirmation of *K. septempunctata*-associated foodborne disease.

We previously established a real-time polymerase chain reaction (PCR) method targeting the small subunit ribosomal DNA (18S rDNA) of *K. septempunctata* as a rapid, accurate, sensitive, and specific diagnostic molecular technique to evaluate the infection levels of this parasite in olive flounders (6). Although this method was useful for detecting the causative foods in *K. septempunctata* foodborne outbreaks (6), it is often difficult to evaluate food samples that are implicated in such outbreaks, as the raw flounder items are frequently entirely consumed. Thus, a suitable, highly sensitive method to analyze patient clinical samples, such as feces and vomitus, is required for investigating outbreaks potentially associated with this novel foodborne pathogen.

The detection of pathogens in feces using light microscopy is a classical laboratory method for diagnosing parasitic cryptosporidiosis and giardiasis (2, 4, 13). However, we were previously unable to detect *K. septempunctata* spores in patient fecal specimens using the formalin-ether sedimentation technique (4, 13), suggesting that the number of intact spores in
each fecal sample was below the detection limit for the microscopic assay. Here, we attempted to apply our highly specific and sensitive real-time PCR method to the detection of *K. septempunctata* in spiked clinical fecal samples. In addition, we compared the relative DNA extraction efficacy of three commercial DNA extraction kits using artificially contaminated fecal samples. Finally, we tested 45 patient samples obtained from 13 foodborne outbreaks associated with the consumption of raw flounder and 41 fecal samples from diarrhea patients epidemiologically unrelated to flounder to confirm whether our detection method can be reliably applied to the investigation of foodborne outbreaks associated with *K. septempunctata*.

**Materials and Methods**

**Real-time PCR assay**

Real-time PCR assays were performed using a previously reported method with minor modifications of the reaction cycling conditions (6). The following cycling conditions were used: 95 °C for 30 s, followed by 45 cycles of amplification at 95 °C for 5 s and 60 °C for 31 s. PCR amplifications were performed using the primers Forward (5'- CGGTCATATCAGCCATGGATAAC
Comparison of three DNA extraction methods using fecal samples artificially contaminated with *K. septempunctata*

Spiking experiments with *K. septempunctata* spores were performed to assess whether our real-time PCR assay was applicable to the detection of *K. septempunctata* DNA in fecal samples and to compare the relative DNA extraction efficiencies among three commercial kits. Three fecal samples (samples A, B, and C) epidemiologically unlinked to the consumption of flounder were spiked with 10-fold dilutions of a *K. septempunctata* spore suspension to obtain concentrations of $1.6 \times 10^4$ and $1.6 \times 10^6$ spores per 1 gram feces for each sample. The spore suspension was prepared from flounders infected with *K. septempunctata* using Percoll (GE Healthcare, Little Chalfont, UK) density gradient centrifugation as previously described (6) and was stored at 4 °C until use.
Three commercial DNA extraction kits, QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), FastDNA SPIN Kit for Soil (MP-Biomedical, Solon, OH), and UltraClean Fecal DNA Kit (MO BIO Laboratories, Inc., Carlsbad, CA), were used to extract DNA from the artificially contaminated fecal samples. To compare the amount of *K. septempunctata* DNA extracted using the three kits, 200 mg of each sample and 200 μl DNA elution buffer were used during the extraction procedure for each kit. Extracted DNA was stored at -20 °C until use. Each DNA extraction was performed in triplicate and real-time PCR was performed in duplicate for each extracted DNA sample. Mean cycle threshold (Ct) values were then compared using the Tukey-Kramer test with the significance level set at *P* < 0.05 to determine the most efficient DNA extraction method.

**Analytical sensitivity of the real-time PCR assay using DNA extracted with the FastDNA SPIN Kit for Soil**

To evaluate the analytical sensitivity of our real-time PCR assay for *K. septempunctata* DNA, two fecal samples (samples D and E) epidemiologically unrelated to the ingestion of flounder were spiked with a *K. septempunctata*
spore suspension at final concentrations of $1.6 \times 10^{-1}$ to $1.6 \times 10^{3}$ spores per 1 gram feces. Total DNA was then extracted from the spiked samples using the FastDNA SPIN Kit for Soil in duplicate. Although the kit protocol recommends the use of 500 mg starting material, we used 300 mg of each spiked sample to avoid sample loss and tube failure caused by overfilling of the matrix tube. Total DNA was eluted with 100 μl DNase/pyrogen-free water. Real-time PCR assays were performed in triplicate using total DNA extracted from both spiked samples. Mean Ct values obtained from the duplicate measurements of samples D and E spiked with equal numbers of *K. septempunctata* spores were compared using the two-tailed Student’s *t* test with a significance level of $P<0.05$.

**Real-time PCR assay evaluation using outbreak patient samples**

Forty-five samples consisting of 43 fecal and 2 vomitus samples obtained from patients involved in 13 foodborne outbreaks occurring between May and November 2011 in western Japan, which were epidemiologically linked to the consumption of raw flounder, were used to evaluate our *K. septempunctata* detection method. Health agencies in each jurisdiction interviewed the patients using standard questionnaires to obtain demographic
and clinical information, and ask about food exposures in the days prior to disease onset. In particular, the investigators asked about the consumption of foods containing raw olive flounder in the 24 h prior to onset, revealing that olive flounder “sashimi” was consumed in all cases, except for one, in which olive flounder “sushi” was consumed. In most patients of these 13 outbreaks, symptoms which were characterized by abdominal pain, diarrhea, nausea, and/or vomiting occurred within 1-14 h. All clinical samples were negative for foodborne bacteria and viruses (enterotoxigenic *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens*, *Salmonella* spp., *Shigella* spp., diarrheagenic *Escherichia coli*, *Campylobacter coli*, *C. jejuni*, *Vibrio cholerae*, *V. parahaemolyticus*, and norovirus genogroup I and II). In 6 out of the 13 outbreaks, $1.3 \times 10^4$ to $2.2 \times 10^7$ *K. septempunctata* spores per gram were detected in the flounders ingested by patients or in the same lot of fish using microscopic morphological examination and our real-time PCR method (6). However, the causative food was not available in the remaining 7 outbreaks. Additionally, 41 fecal samples from patients with gastrointestinal disease in several foodborne outbreaks epidemiologically unrelated to *K. septempunctata* (17, 7, and 17 patients in *Salmonella enterica* serovar Enteritidis, *C. coli* and *C. jejuni*).
jejuni, and norovirus outbreaks, respectively) were also tested. Three-hundred milligrams of each sample was subjected to DNA extraction using the FastDNA SPIN Kit for Soil.

To confirm that no false-positive results were detected, the real-time PCR products were electrophoresed for 30 min in a 2.0% agarose gel at 100 V and visualized by staining with ethidium bromide and viewing under UV light for confirming the presence of amplicons of the expected size. Additionally, a nested PCR assay was conducted using the following forward and reverse flanking primers: (forward-114) 5’-ATGGATAACTGTGGTAAATCTAGCTAATAC-3’ and (reverse-200R) 5’-CCAGTTGGTCTAGCTAATATGC-3’. The nested PCR reaction mixture was prepared using PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Piscataway, NJ, USA) containing 5 pmol of each primer and 2.5 μl of a 1/100 dilution of the real-time PCR products in a total volume of 25 μl. Amplification conditions involved an initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, followed by a final elongation step at 72 °C for 5 min. The amplified products were electrophoresed and visualized as described above.
To exclude the possibility that inhibition of the real-time PCR reaction led to false-negative results, separate samples were spiked with a 1/100 volume of a *K. septempunctata* 18S rDNA plasmid solution (1.1 × 10³ copies/μl) and run in parallel with unspiked samples. The plasmid DNA solution was prepared as described previously (6).

Fisher’s exact test with a significance level of *P* < 0.05 was used to assess the association between the real-time PCR detection rate and the total length of time from causative food consumption to specimen collection.

Results

Comparison of three commercial DNA extraction kits for use in real-time PCR

Table 1 shows the mean Ct values of real-time PCR reactions performed with DNA extracted from three spiked fecal samples (samples A, B and C) using three commercial DNA extraction kits. In assays using samples spiked at the lowest level (1.6×10⁴ spores/g), the lowest Ct values were obtained from DNA samples extracted by the FastDNA SPIN Kit for Soil for all
three fecal samples. Moreover, the lowest Ct values were also obtained for assays performed using DNA extracted with the FastDNA SPIN Kit for Soil in 2 of the 3 fecal samples spiked with 1.6×10^6 spores/g. These data suggested that the FastDNA SPIN Kit for Soil was the most efficient of the three commercial kits for extracting *K. septempunctata* DNA from the spiked fecal samples. Therefore, this kit was selected for use in all subsequent experiments.

**Analytical sensitivity of the real-time PCR assay with spiked fecal samples**

To evaluate the analytical sensitivity of the real-time PCR assay developed for *K. septempunctata* detection, two fecal samples (samples D and E) spiked with 1.6×10^{-1} to 1.6×10^3 spores of *K. septempunctata* per gram were analyzed (Table 2). The mean Ct values for the real-time PCR reactions were determined using a threshold line of 0.35. Although amplification products were not obtained for samples spiked with 1.6×10^{-1} or 1.6×10^3 spores/g, the mean Ct values of samples D and E, which were spiked with 1.6×10^1 spores/g, were 36.83 (±0.36) and 39.75 (±1.24), respectively, suggesting that the detection limit of the real-time PCR assay was 1.6×10^1 spores per gram of feces. Based
on these findings, we concluded that the patient fecal samples were positive for *K. septempunctata* if the Ct value was equal to or less than 41 cycles, when the threshold line of the real-time PCR assay was set at 0.35 for fluorescent intensity.

In addition, significant differences in the Ct values were detected between the two fecal samples spiked with $1.6 \times 10^1$, $1.6 \times 10^2$, and $1.6 \times 10^3$ spores/g (two-tailed Student’s *t* test; *P*<0.05).

Detection of *K. septempunctata* in clinical samples from patients associated with outbreaks due to the consumption of raw flounder

To evaluate the sensitivity of our real-time PCR assay for the detection of *K. septempunctata* in clinical samples, 43 fecal and 2 vomitus samples obtained from 13 foodborne outbreaks associated with the consumption of flounder were tested. Of 43 fecal samples, positive real-time PCR results for *K. septempunctata* were detected in 21 samples. In addition, both vomitus samples gave positive results in the assay. Single PCR products of the expected size (159 bp) were amplified from all 23 positive samples and the Ct values of the positive clinical samples ranged from 30.78-40.69. Moreover,
nested PCR products were also obtained for all real-time PCR-positive samples. Eventually, *K. septempunctata* 18SrDNA was detected in at least one clinical sample from each of the 13 outbreaks. Importantly, no false-negative results were detected, and no PCR products were amplified from the 41 fecal samples obtained from patients involved in the foodborne outbreaks epidemiologically unrelated to *K. septempunctata*.

Of 40 fecal samples whose sampling dates were known, the detection rate (63.0%) of *K. septempunctata* in the 27 fecal samples collected within 2.5 days of the consumption of flounder was significantly higher than that (23.1%) in the 13 fecal samples collected later than 2.5 days after the consumption (Fisher's exact test, *P* < 0.05).

**Discussion**

In the present study, we demonstrated that our previously established real-time PCR assay was applicable for the detection of *K. septempunctata* DNA in clinical fecal samples. Moreover, it was determined that the FastDNA SPIN Kit for Soil more efficient extracted *K. septempunctata* DNA from artificially contaminated fecal samples than the QIAamp DNA Stool Mini Kit and
UltraClean Fecal DNA Kit. Notably, our results showed that increasing the duration between the intake of infected olive flounder and sample collection affected the ability of our real-time PCR assay to detect *K. septempunctata* in feces. Despite this limiting factor, the presently described method would be useful for laboratory investigations of foodborne outbreaks caused by this novel pathogen.

To develop a detection method with higher sensitivity for *K. septempunctata* DNA, we first compared the relative DNA extraction efficiencies from fecal samples artificially contaminated with *K. septempunctata* spores using three commercial kits. We found that the FastDNA SPIN kit for Soil, which has been used to directly isolate PCR-ready genomic DNA of microorganisms not only from soil and sediments, but also from feces (1, 9), displayed the highest efficiency in almost all assays. Therefore, this kit was used with our real-time PCR assay for detecting *K. septempunctata* in clinical samples of outbreak patients.

The detection limit of our real-time PCR assay used in conjunction with the FastDNA SPIN kit for Soil in the spiking experiments was $1.6 \times 10^1$ spores per gram of feces. As 300 mg fecal sample spiked with $1.6 \times 10^1$ *K.
septempunctata spores per gram theoretically contains approximately 5 spores, and we previously showed that a single spore of *K. septempunctata* contains at least approximately $3.0 \times 10^2$ of 18S rDNA copies (6), the real-time PCR detectable limit in the spiking experiment was approximately $3.0 \times 10^1$ of 18S rDNA copies per reaction. This detection limit was nearly equal to that of a real-time PCR assay performed using plasmid DNA as a control ($1.1 \times 10^1$ copies of *K. septempunctata* 18S rDNA per reaction) in our previous study (6). These data suggest that presently described real-time PCR assay for the detection of *K. septempunctata* in fecal samples has a similar sensitivity to that used for plasmid-encoded *K. septempunctata* 18S rDNA. However, the mean Ct values for two samples that samples that were spiked with equal numbers of *K. septempunctata* spores ($1.6 \times 10^1$, $1.6 \times 10^2$, and $1.6 \times 10^3$ spores/g) significantly differed, suggesting that the consistency (e.g., solid, soft, or watery) and composition of feces might affect the efficiency of DNA extraction or amplification efficiency of the real-time PCR assay.

Of 45 clinical samples collected for the investigation of foodborne outbreaks associated with consumption of raw flounder, 2 vomitus and 21 fecal samples were positive for *K. septempunctata* DNA by our developed real-time
PCR method. Although the Ct values of all 23 positive samples exceeded 30 cycles, single PCR products of the expected size were amplified from all positive samples and a nested PCR assay confirmed that all amplicons were specific for *K. septempunctata* 18S rDNA. These data indicate that no false-positive results were obtained in the detection assays, and that only a small amount of detectable *K. septempunctata* DNA was present in feces and vomitus from outbreak patients. Additionally, as no PCR products were amplified from 41 fecal samples obtained from foodborne outbreaks epidemiologically unrelated to *K. septempunctata*, our real-time PCR assay using fecal matter appears to maintain the high specificity previously demonstrated for flounder tissue.

Our case file analyses of 13 foodborne outbreaks investigated in this study revealed that the median incubation time in all cases was 7 h or less. Additionally, transient diarrhea and/or vomiting were the characteristic symptoms of patients. Together, the epidemiological data indicated that *K. septempunctata* was likely the etiological agent in these outbreaks (7). As predicted, in 6 of the 13 outbreaks, *K. septempunctata* spores were detected in the implicated olive flounder remnants or the same lot samples. However,
because the food remnants were not obtained in the other seven cases, the causative food was not conclusively identified and other supporting evidence for the link between K. septempunctata and these outbreaks was required. As a result of the analyses performed here, in which K. septempunctata DNA was detected in at least one clinical sample from each of the seven cases, we have provided strong evidence that this pathogen was the causative disease agent in these seven outbreak cases. The present findings also indicate that our real-time PCR method using clinical samples is an effective tool for investigating K. septempunctata foodborne outbreaks.

The significant decrease in the detection rate of K. septempunctata in patient fecal specimens collected later than 2.5 days after the intake of causative food indicates that the length of the period from the time of consumption to specimen collection is an important factor for the successful detection of K. septempunctata using this assay. Myxosporean parasites, such as well-studied Myxobolus cerebralis (8, 14), typically have a two-stage life cycle alternating between fish and annelids, although little is known about the life cycle of K. septempunctata within or outside of olive flounders. K. septempunctata outbreak patients generally recover within 24 h of the onset of
symptoms and fluid accumulation induced by *K. septempunctata* spores does not persist longer than 4 h in the suckling mouse test (7). Based on these facts, we hypothesize that *K. septempunctata* likely has a similar life cycle to other myxosporean parasites, and that mammals, such as humans and suckling-mice, may have no association with the life cycle of this parasite. Therefore, *K. septempunctata* may not be able to colonize and proliferate in the mammalian gastrointestinal tract and be rapidly excreted in the feces. Our finding that *K. septempunctata* is predominantly detected in feces during only a short period in comparison with other diarrheagenic parasites, which infect and proliferate in the gastrointestinal tract, may support this hypothesis (2, 3, 11). However, further studies, such as investigating the mechanisms underlying the diarrheal activity induced by *K. septempunctata* in humans or suckling-mice, are required to confirm this hypothesis and shed light on the life cycle of this emerging pathogen.

As the consumption of raw fish is a traditional Japanese food custom, the confirmation that olive flounder infected with *K. septempunctata* may cause foodborne illness and outbreaks represents a major public health concern in Japan. Moreover, as Japanese foods prepared with raw fish, such as sushi and
sashimi, are also popular in many developed countries, that threat of foodborne disease caused by *K. septempunctata* also exists outside of Japan. Thus, we anticipate that our presently described method for the sensitive detection of *K. septempunctata* in clinical samples may be an effective tool for the investigation of both domestic and international outbreaks caused by this novel pathogen.

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### References


Table 1. Mean Ct values by three commercial DNA extraction kits

<table>
<thead>
<tr>
<th>Concentration of spiked <em>K. septempunctata</em> spores in fecal samples</th>
<th>Low (1.6×10^4 spores/g)</th>
<th>High (1.6×10^6 spores/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QIA stool</td>
<td>Fast Prep for Soil</td>
</tr>
<tr>
<td><strong>Sample A</strong></td>
<td>37.65 ± 0.747^c</td>
<td>27.55 ± 0.286^a</td>
</tr>
<tr>
<td><strong>Sample B</strong></td>
<td>37.15 ± 0.435^c</td>
<td>29.40 ± 2.264^a</td>
</tr>
<tr>
<td><strong>Sample C</strong></td>
<td>36.96 ± 0.422^c</td>
<td>27.75 ± 0.108^a</td>
</tr>
</tbody>
</table>

Superscript letters indicate the DNA extraction efficiency in each experiment, from high (A) to low (C), as determined using the Tukey-Kramer test with a significance level (P) of 0.05.
Table 2. Mean Ct values in spiking experiments used to evaluate the analytical sensitivity of the real-time PCR assay for *K. septempunctata* in fecal samples

<table>
<thead>
<tr>
<th>Inoculum dose (spores/g)</th>
<th>1.6×10^{-1}</th>
<th>1.6×10^{0}</th>
<th>1.6×10^{1}</th>
<th>1.6×10^{2}</th>
<th>1.6×10^{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample D</td>
<td>-</td>
<td>-</td>
<td>36.83 (± 0.36)^a</td>
<td>33.91 (± 0.64)^b</td>
<td>30.16 (± 0.35)^c</td>
</tr>
<tr>
<td>Sample E</td>
<td>-</td>
<td>-</td>
<td>39.75 (± 1.24)^a</td>
<td>36.61 (± 0.55)^b</td>
<td>32.19 (± 0.29)^c</td>
</tr>
</tbody>
</table>

Superscript A-C denotes pairs where the difference in mean Ct values was statistically significant (*P*<0.05) by two-tailed Student's *t*-tests.

Data are presented as the mean ± standard deviation. -, undetermined.